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Preclinical report

8-Cl-cAMP and tiazofurin affect vascular endothelial growth factor production and glial fibrillary acidic protein expression in human glioblastoma cells

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Compounds that could block tumor angiogenesis and induce tumor cell differentiation in malignant gliomas represent a very valuable tool in anticancer treatments. In this paper, we demonstrate that more selective drugs, which interfere with specific cellular targets, could treat glioma more effectively. 8-Cl-cAMP and tiazofurin (TR) are site-specific analogs that selectively inhibit PKAI and IMP dehydrogenase, are directly involved in cell proliferation and apoptosis, and mediate the mitogenic effects of different oncogenes and growth factors. In this study, we have examined influence of 8-Cl-cAMP and TR on the production of an angiogenic factor [vascular endothelial growth factor (VEGF)] by human glioblastoma U251 MG cells, as well as their influence on the expression of a differentiating marker [glial fibrillary acidic protein (GFAP)]. Using a cell proliferation assay, VEGF enzyme-linked immunoassay and GFAP immunocytochemistry we demonstrated the effects of these compounds. Our results demonstrate that 8-Cl-cAMP and TR decrease VEGF production by U251 MG cells, and that under the influence of both agents these cells increase GFAP expression and change their morphology, becoming more differentiated. These findings also suggest that 8-Cl-cAMP and TR may have potential for further investigation of their antiangiogenic and differentiational role in malignant disease such as human gliomas. [© 2000 Lippincott Williams & Wilkins.]

Key words: 8-Cl-cAMP, differentiation, glial fibrillary acidic protein, human malignant glioma, tiazofurin, vascular endothelial growth factor.

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Introduction

Gliomas are the most common primary tumors in the central nervous system and glioblastoma multiforme is the most advanced stage.[1] Although surgery, radiotherapy and chemotherapy can prolong survival, these treatments have a poor therapeutic prognosis and high toxicity.

8-Cl-cAMP (one of the C-8 adenosine-3′,5′-monophosphate analogs) specifically down-regulates type I protein kinase A, a signaling protein directly involved in cell proliferation and neoplastic transformation, and causes growth inhibition in a variety of human cancer cell types.[1,2] The growth inhibition induced by 8-Cl-cAMP was accompanied with various effects, including biochemical and morphological changes, differentiation, and reverse transformation.[3]

Tiazofurin [TR; 2-β-D-ribofuranosyl thiazole-4-carboxamide] is a potent inhibitor of inosine monophosphate dehydrogenase (IMPDH) and a novel anticancer drug. The inhibition of IMPDH leads to decreased guanosine triphosphate (GTP) concentration, causing inhibition of DNA and RNA biosynthesis and cell proliferation.[4] More and more data are available showing that TR, besides its antiproliferative effect, also exerts a profound effect on several other events of cell life, such as apoptosis and cell differentiation.[4–6]

Vascular endothelial growth factor (VEGF) is one of the most important angiogenic factors. It is secreted by tumor cells and induces mitogenesis of capillary endothelial cells.[7] Therefore, VEGF plays a major role in the biological behavior and morphological findings of tumor neovascularization. VEGF production can be enhanced in response to hypoxia and epidermal growth factor (EGF).[8,9] EGF stimulates the proliferation of EGF receptor-positive malignant glioma cells with attendant induction of VEGF production.[9] Hence,

VEGF is considered as an indicator of malignancy potential of astrocytic tumors.[10] Malignant gliomas are an attractive model for the investigation of the role of VEGF in tumor angiogenesis. They are among the most dramatically neovascularized neoplasms with respect to vasoproliferation, endothelial cell cytology and endothelial cell hyperplasia.[11] Compounds that block VEGF production in malignant gliomas could represent a very valuable tool in anticancer treatment.

The studies of the expression of cell type-specific, differentiation-related proteins in malignant gliomas might provide important insights into manipulating them to modulate their behavior. Glial fibrillary acidic protein (GFAP) is a major constituent of glial cytoplasmic filaments considered specific for mature cells of astroglial lineage.[12] GFAP monoclonal antibodies are the most commonly used markers for the identification of astrocytes and glia-related neoplasms.[13] Glioblastoma multiforme, the most malignant glioma, is rapidly growing, highly invasive and often associated with low GFAP expression. In contrast, low-grade astrocytomas are usually associated with high GFAP expression.[14] Thus, the number of $GFAP^+$ cells in glial tumors is inversely related to their malignant behavior. The presence of both $GFAP^+$ and $GFAP^-$ cells in a single tumor is evidence of the heterogeneity of malignant gliomas. Heterogeneous tumors are composed of multiple cell populations that may have differing growth rate and invasive potentials. The $GFAP^-$ tumor cells are believed to outgrow their $GFAP^+$ counterparts.[15] Therefore, compounds that could induce expression of GFAP in tumor cells have a potential to control tumor growth and invasive potential.

In this study, we investigated the effects of 8-Cl-cAMP and TR on the production of VEGF secretion and induction of differentiation of malignant glioma cells.

Materials and methods

Cell culture

U251 MG glioma cell line was provided by Dr Martin Haas (University of California San Diego, Department for Biology, La Jolla, CA). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with heat-inactivated calf serum (10% v/v), L-glutamine (2 mM), penicillin (50 UI/ml) and streptomycin (50 μg/ml) (all from ICN Pharmaceuticals, Costa Mesa, CA). Also, they were cultured in astrocyte basal medium (ABM) supplemented with FBS (5% v/v), progesterone (25 μg/ml), transferrin (50 mg/ml), insulin (10 mg/ml), human recombinant epidermal growth factor (hEGF; 10 μg/ml), gentamicin (50 mg/ml) and amphotericin-B (50 μg/ml) (all from Clonetics Walkersville, MD). Cells were subcultured at 72 h intervals using 0.25% trypsin/EDTA (Clonetics) and seeded into fresh medium at 1:15. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

8-Cl-cAMP and TR

8-Cl-cAMP and TR were synthesized in R & D (ICN, Belgrade, Yugoslavia).

Cell proliferation analysis

Cells were seeded into 96-well plates at 4×10^4 cells/well and treated with different concentrations of 8-Cl-cAMP and TR. The plates were incubated for 68 h, after which [^{3}H]thymidine (ICN) was added to each well to a final concentration of 1 μCi/ml. Cells were incubated for 4 h to allow incorporation of [^{3}H]thymidine into cellular DNA. They were then lysed with 0.25% trypsin/EDTA (Clonetics) for 15 min. Cell lysate was harvested (Tomtec Harvester 96®, Wallac, Turku, Finland) and counted using 1205 Betaplate Liquid Scintillation Counter (Wallac). Incorporation of [^{3}H]thymidine into cell DNA was expressed as c.c.p.m. (corrected c.p.m.), average $\pm$ SD of at least triplicate determinations.

Measurement of secreted VEGF

U251 MG cells were seeded into 100×20 mm Petri dishes (Corning, Acton, MA) at 5×10^5 cells/dish, and treated with 8-Cl-cAMP and TR. Medium with secreted VEGF was collected after 72 h of incubation, 20 times diluted and levels of VEGF were measured by enzyme-linked immunoassay (Quantikine™; R & D Systems, Minneapolis, MN) using 96-well microtiter plates in accordance with the manufacturer's instructions. Antibodies were directed against human recombinant $VEGF_{165}$. The immobilized antibody was monoclonal, while the second horseradish peroxidase-conjugated antibody was polyclonal. The level of VEGF was calculated using a standard curve obtained with human recombinant $VEGF_{165}$ (from 15.6 to 2000 pg/ml). All determinations were performed in triplicate and the results were expressed as ng/ml.

Immunocytochemistry

Cells were seeded into four-well chamber slides (Nunc Nalgene, Rochester, NY) at 2×10^4 cells/well and treated with 8-Cl-cAMP and TR for 72 h. Cells grown in

chamber slides were fixed in 4% paraformaldehyde and cell membrane was permeabilized with acetone at −20°C. After 20 min of incubation with normal serum that block non-specific binding (Vectastain®, ABC kit; Vector, Burlingame, CA) at room temperature, cells were incubated with monoclonal anti-GFAP (×100; Sigma, St Louis, MO) for 30 min at 37°C. Secondary biotinilizated antibody (Vectastain® was applied for 30 min at room temperature. Incubation with ABC reagent lasted for 60 min at room temperature. Immunocytochemical reaction was visualized with 3,3′-diaminobenzidine (DAB/metal concentrate; Boehringer Mannheim, Mannheim, Germany), 10 times diluted in peroxidase buffer (Boehringer Mann-heim). The slides were mounted with Kanadabalsam (Merck, Darmstadt, Germany). Immunostained cells were examined under a light microscope (Olympus, Hamburg, Germany) and classified as GFAP(−), GFAP(+), GFAP(++) and GFAP(+++). At least 1000 cells were counted for untreated cells, as well as for cells treated with 10 μM of 8-Cl-cAMP and 15 μM of TR.

Morphologic signs of differentiation

The criteria for cell differentiation were the following: (i) loss of triangular or polygonal shape, which is characteristic for U251 MG cells, (ii) formation of long cell processes, which are typical for differentiated glial cells, and (iii) resemblance of mature astrocytes morphology.

Method of data analysis

All results were expressed as the averages ± SD. The Student *t*-test (two-tailed) was utilized to compare and evaluate the statistical significance of the data. A p value of less than 0.05 was considered to indicate a significant difference.

Results

In this study we compared the effects of 8-Cl-cAMP and TR on the cell proliferation of U251 MG glioma cells. 8-Cl-cAMP and TR have decreased [^{3}H]thymidine incorporation in U251 MG cells in a dose-related fashion (Figure 1). Untreated U251 MG cells have shown a 96% increase in [^{3}H]thymidine incorporation when they were cultured in astrocyte growth medium (AGM; data not shown). Despite enhanced growth of those cells in AGM when compared with growth in DMEM, the effect of 8-Cl-cAMP and TR remained in a dose-related manner (Figure 1). After 72 h of treatment, the inhibitory concentrations (IC_{50}) for 8-Cl-cAMP were 4.1 and 3.9 μM, while they were 16 and 20.2 μM for TR in DMEM and AGM, respectively. Lower concentrations of 8-Cl-cAMP have shown a stronger effect on cell proliferation in comparison with TR. 8-Cl-cAMP had a similar effect in DMEM and AGM, by reaching a plateau at 10 μM. The lowest concentration of TR that has shown a substantial decrease in [^{3}H]thymidine incorporation was 15 μM.

VEGF is one of the most important angiogenic factors secreted by tumor cells. Therefore, we wanted to determine the effects of 8-Cl-cAMP and TR on VEGF secretion of U251 MG glioma cells. $VEGF_{165}$ levels were quantified by ELISA in cell culture media conditioned for 3 days. Untreated U251 MG cells secreted 5.49 ng/ml VEGF in DMEM, while the level of VEGF secretion was 7.88 ng/ml (44% increased) when they were cultured in AGM (Figure 2). In further experiments, we have used 10 μM of 8-Cl-cAMP and 15 μM of TR for treatment. Those concentrations

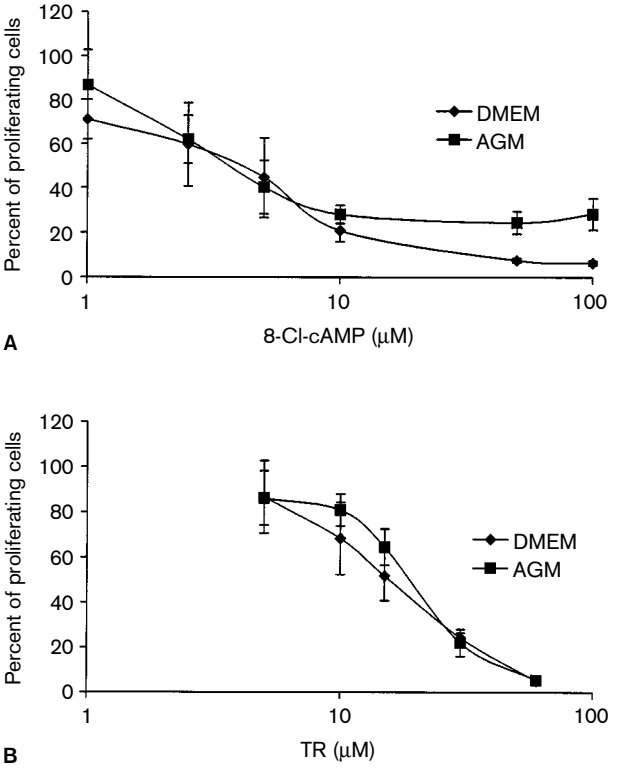

Figure 1. The effect of different concentrations of 8-Cl-cAMP and TR on the rate of [^{3}H]thymidine incorporation in U251 MG human glioma cells, after 72 h of treatment. (A) Effect of 8-Cl-cAMP on U251 MG cells cultured in DMEM and AGM media. (B) Effect of TR on U251 MG cells cultured in DMEM and AGM media. The percent of proliferating cells (P) was obtained with next equation: $P=C/C_0$, where C_0 represents c.c.p.m. of untreated cells and C represents c.c.p.m. of cells treated with different concentrations.

decreased VEGF production in both media, with more potency in DMEM (Figure 2). The tested concentration of 8-Cl-cAMP decreased VEGF production by U251 MG cells 74% in DMEM, while the same concentration decreased VEGF production in AGM only 43%. In the case of TR, VEGF production was decreased 50% in DMEM and 22% in AGM (Figure 2).

Comparing the results obtained with [^{3}H]thymidine incorporation and measurement of secreted VEGF, we have shown that the percent of proliferating cells and cells that secrete VEGF, treated with 8-Cl-cAMP and TR, were correlated when cultured in DMEM (Table 1). However, VEGF secretion affected with 8-Cl-cAMP and TR was elevated in comparison with the percent of proliferating cells cultured in AGM (Table 1).

In the next series of experiments we examined whether the antiproliferative effects of 8-Cl-cAMP and TR on human glioma cells are correlated with morphological changes in these cells. The population of untreated glioma cells is heterogenic with triangular or polygonal cells (Figure 3A). Some of the treated cells resembled the morphology of mature astrocytes by forming long cell processes, which are typical of differentiated glial cells (Figure 3B and C). Under the influence of both drugs, U251 MG cells changed their morphology, becoming more differentiated (Figure 4). The percent of differentiated cells after treatment with 8-Cl-cAMP was increased 34% in comparison with untreated cells, while TR increased the portion of differentiated cells 12% (Figure 4).

Immunohistochemical analysis with the glia cell marker GFAP confirmed these data. The degree of GFAP expression was markedly elevated in treated cells while the untreated cells demonstrated faint GFAP expression throughout the cytoplasm (Figure 3). The number of positive staining cells and intensity of staining were increased. GFAP staining was characterized as negative (−), positive (+), (++) and (+++). GFAP staining was considered genuine because control cultures treated with PBS instead of the primary antibody demonstrated little or no specific staining. 8-Cl-cAMP and TR decreased the portion of GFAP(−) cells in comparison with untreated cells (Table 2). Both agents increased the portion of GFAP(++) and GFAP(+++) cells, with 8-Cl-cAMP being more active on increasing the portion of GFAP(+++) cells (Table 2).

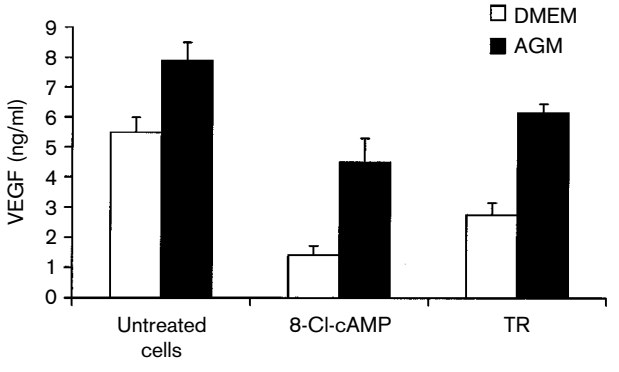

Figure 2. VEGF secretion of U251 MG human glioma cells in DMEM and AGM media after treatment with 8-Cl-cAMP and TR analyzed by ELISA and calculated per ml cells. $VEGF_{165}$ was measured quantitatively using a commercially available ELISA performed according to the manufacturer's instructions.

Discussion

The strong biological potential of 8-Cl-cAMP and TR is evident from the fact that their inhibitory effect on human glioma U251 MG cell line proliferation was not diminished in AGM despite rapid cell growth in this medium (Figure 1). Untreated U251 MG cells have shown 96% increased proliferation when they were cultured in AGM in comparison with DMEM. The proliferation of cells was additionally induced with growth factors and hormones supplied in AGM.

One of the growth factors contained in AGM was EGF, a major positive regulator of VEGF *in vitro*.[9] In addition, EGF is produced autocrinaly by many tumor types including human gliomas. Considering this, the stronger potential of 8-Cl-cAMP and TR for blocking VEGF secretion was expected in DMEM, as we have shown (Figure 2). The portion of proliferating cells

Table 1. Percent of proliferating cells and percent of VEGF secretion affected by 8-Cl-cAMP and TR compared in two different media

	DMEM		AGM	
	Proliferating cells	VEGF secretion	Proliferating cells	VEGF secretion
8-Cl-cAMP	20.8±7.1	25.8±5.4	28.0±4.3	57.0±10.1
TR	51.9±11.4	50.1±7.2	64.6±8.2	77.8±3.8

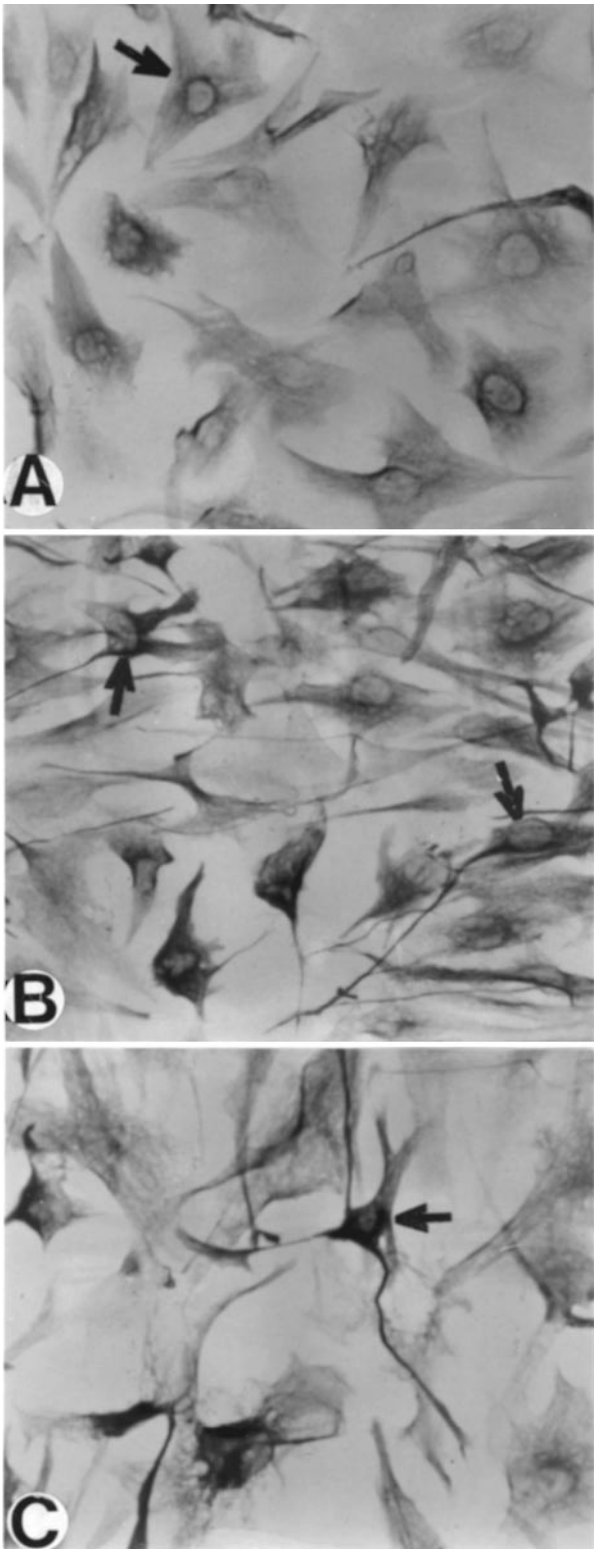

Figure 3. GFAP expression visualized with 3,3′-diaminobenzidine. Magnification ×600. (A) Untreated U251 MG cells with characteristic triangular and polygonal morphology. Arrow indicates a typical U251 MG cell without cytoplasmatic processes. (B) U251 MG cells treated with 10 μM of 8-Cl-cAMP exhibit enhanced expression of GFAP and increased extension of cell processes. Arrow indicates a GFAP-positive cell (+++) with signs of morphological differentiation. (C) U251 MG cells treated with 15 μM of TR exhibit enhanced expression of GFAP and increased extension of cell processes. Arrow indicates GFAP-positive cell (+++) with signs of morphological differentiation.

and the portion of cells that secrete VEGF, treated with both agents, were correlated in DMEM. In contrast, the portion of cells that secrete VEGF was elevated in comparison with the portion of proliferating cells in AGM (Table 1). This could be due to EGF induction of VEGF that could not be exceeded with 8-Cl-cAMP and TR despite their strong antiproliferative effect.

Since it was shown that with increasing astrocytic anaplasia there is progressive loss of GFAP production,[16] GFAP has been considered a reliable marker of differentiation for normal astrocytes and for tumors of astrocytic lineage. A few permanent cell lines originated from human gliomas express GFAP. However, 80% of U251 MG cells intrinsically express GFAP.[17] According to our study, 72.4% of untreated U251 MG cells were GFAP-positive. Under the influence of 8-Cl-

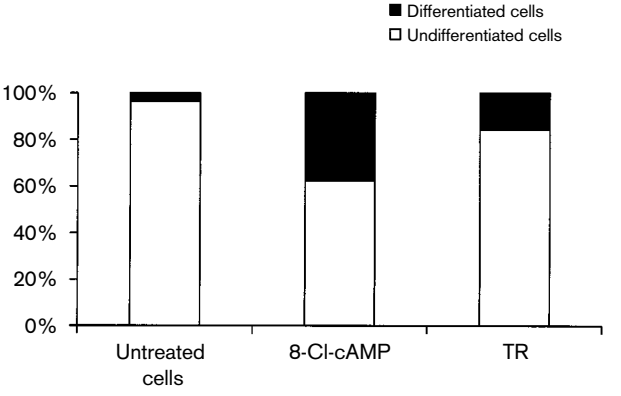

Figure 4. Cell differentiation induced by 8-Cl-cAMP and TR. The percent of differentiated cells after treatment with 8-Cl-cAMP and TR was calculated in comparison with untreated cells.

Table 2. Percent of GFAP expression in cells treated with 8-Cl-cAMP and TR

	GFAP (−)	GFAP (+)	GFAP (++)	GFAP (+++)
Untreated cells	27.6	50.4	18.4	3.5
8-Cl-cAMP	4.2	45.5	26.7	23.3
TR	15.7	44.1	33.2	7.0

cAMP and TR, the number of positive cells was elevated to 95.8 and 84.3% of cells, respectively (Table 2).

Associated with the enhanced expression of GFAP was the increased extension of cell processes, one more sign of glial cell differentiation. Untreated triangular and polygonal U251 MG cells were stained with GFAP, indicating positive filaments extending from the perinuclear zone (Figure 3A). Treated cells have shown intensive positive GFAP staining in their long processes (Figure 3B and C). 8-Cl-cAMP and TR changed the morphology of U251 MG cells by making them more differentiated toward the mature astrocytes phenotype.

The failure of human malignant glioma to respond to current treatment protocols requires development of novel treatment strategies. The fact that 8-Cl-cAMP and TR can act as VEGF secretion suppressors in U251 MG human glioma cells is interesting for the design of future antiangiogenic therapeutic strategies. Furthermore, as both agents at the same time induce cellular differentiation, it could be one more argument for further investigation of their antiangiogenic and differentiational role in human malignant gliomas.

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